THE DEVELOPMENT OF CHEMICAL RESISTANCE IN PLANT PATHOGENIC FUNGI

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AN ABSTRACT OF A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PLANT PATHOLOGY IN THE GRADUATE COLLEGE OF THE UNIVERSITY OF ILLINOIS, 1950

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I. Introduction

The principal objective of this study was to attempt to develop resistance strains of *Sclerotinia fructicola, Glomerella cingulata,* and *Penicillium expansum* to the chemicals Cu_2O, $CuSO_4$, $CuCl_2$, Bordeaux, Bioquin-1, Bioquin, ferbam, Puratized, and Isothan DL-1. It has been possible to develop resistance in the three fungi to one or more of the above chemicals. At the same time a number of mutants developed which were hardly recognizable as coming from the parent strain. Such resistance may have a wide application to both plant and animal pathology and in the study of fungal diseases that require protectant or eradicant fungicides for control.

A review of literature indicated that there were a few instances of the development of resistance in fungi to certain chemicals in which pure or nearly pure chemicals were used.

In the development of resistance a number of questions presented themselves: 1. Can resistance be developed in fungi to commercial fungicides? 2. Can mass inoculum be used in developing resistance? 3. Can the effects of the fungicides be entirely overcome? 4. Will the changes that occur be permanent? 5. Will the viability of these organisms be changed? 6. Can the experimental work give some evidence that the fungi used will produce new strains in the field?

II. Methods

The two methods used to induce resistance in the fungi were essentially alike. Method I was a plate-to-plate transfer involving masses of spores and later plugs of mycelium. The cultures used were designated as: R for the fungus constantly exposed to the chemicals, C for the strains exposed once at each transfer, and C-1 for the control never exposed to chemicals.

The first 7 chemicals above were used in the mass spore test while Puratized and Isothan DL-1 were included in the plug tests. The steps involved in Method I were: 1. Preparation of a whole-wheat agar medium. 2. One half cc. of a counted spore suspension from week old cultures was added to the plates. 3. Measured quantities of sterile chemicals were added to the media, which was poured into the plates containing the spores. Duplicate cultures of the fungi were grown at

each transfer on each of 2-3 concentrations for the R and C strains. 4. The R strains were transferred from plates containing the highest concentration of chemical on which there was a fair production of spores. After a number of transfers the mass spore tests were discontinued because some strains of the fungi exposed to the chemicals failed to produce spores. The time for each transfer depended on the amount of growth of the R strain and varied from 10 days to several weeks. Following the mass spore tests 5 mm. plugs of growth were transferred to plates containing the chemicals. Measurements of the radii were made every 2 days until 4 measurements had been recorded. 5. After numerous changes had occurred in the R strains, transfers were made to an inhibitor-free medium to determine whether or not the changes were permanent. Growth measurements of the radii were made for 10 successive transfers of the R and C-1 strains. 6. Spore germination tests were made of the 2nd, 3rd, 4th, and 5th transfers of this same series. 7. After the 10th transfer to inhibitor-free medium apples were inoculated for viability tests.

By using two methods it was possible to show that some of the results could be repeated to prove that the changes did not occur by chance alone.

Method II was a flask-tube-plate-flask test. The chemicals used were: Cu_2O, Bordeaux, Bioquin-1, ferbam, Puratized, and Isothan DL-1. The media used were whole-wheat agar as in Method I, Czapek's solution medium in flasks, and tubes of whole-wheat-dextrose medium. Steps in Method II were: 1. Single spore isolations were made from spore germination tests in each of the chemicals given above. The spores were put on plates of whole-wheat agar and grown for one week. Then one plug from the R and C strains was added to each of 2 flasks of Czapek's for a 48 hour rejuvenation period. The flasks were put on a shaker devised for this test. 2. Measured quantities of the chemicals were added to the flasks following the rejuvenation period. Two concentrations were used for each of the R and C strains. 3. The cultures were grown for one week then transfers of the C-1 strains were made to the tubes of broth for another 48 hour rejuvenation period. 4. Transfers were made to the plates from the tubes. Two colonies were put on each plate. The plates were made the same as in Method I plug test except only 2 plates were made for each of 2 concentrations. Growths were measured and recorded. Plugs were transferred to the flasks repeating the cycle. 5. The contents of the flasks, after transfers to the tubes were filtered, washed, put into Gooch crucibles, dried, weighed, then ashed and reweighed. The pH of the solution in the flasks was determined after growth.

III. Results

Results of Method I show that *Sclerotinia* apparently developed resistance in the mass spore tests at or below the concentrations given: 280 ppm Cu_2O, 650 ppm $CuSO_4$, 25 ppm Bordeaux, 2 ppm Bioquin, and 20 ppm ferbam. This was shown when the R strains of the first plug transfer grew faster than the C strain. Generally, higher concentrations of the chemicals were used in the plug tests than in the spore tests.

Sclerotinia developed resistance in the plug tests to a significant degree on the following chemicals at or below the concentrations given: 400 ppm Cu_2O, 650 ppm $CuCl_2$, 168 ppm Bordeaux, 6 ppm Bioquin, 60 ppm ferbam. The chemicals $CuSo_4$ and $CuCl_2$ stimulated microspore formation at 500-600 ppm. Germination of microspores was induced by $CuCl_2$ at 600 ppm in the R strain. No microspores formed on the Puratized media. All the chemicals reduced macrospore formation except ferbam and Isothan DL-1 which increased macrospore formation. In spore germination tests the spores from the R strain of Cu_2O medium developed resistance. The LD 97 and LD 8 for the C-1 and R strains respectively was 30 ppm.

When transfers were made to the inhibitor-free medium all R strains apparently reverted to the C-1 control except Puratized R strain which reverted only after it was passed through apple. The average growth rates of the R and C-1 strains including the least significant difference are shown in the table:

Average Growth Rate of the R and C-1 Strains at the End of 6-8 Days from 10 Successive Transfers to Inhibitor-free Medium

Chemical	Sclerotinia	Glomerella	Penicillium
Cu_2O	36	35.5	24.5
$CuSO_4$	36.5	23	22
$CuCl_2$	34	31	24
Bioquin	39.5	23.5	29.5
Bioquin-1	37	25	18
Bordeaux	41	29	25
ferbam	37.5	25.5	26
Puratized	20	27.5	25.5
Isothan DL-1	39	30	26
C-1	38	28	26.5
LSD	4.26	4.31	4.68

Morphological changes were slight in *Sclerotinia*. No sectoring in the strains occurred over a period of 12 months with 30 transfers.

Glomerella developed resistance in the mass spore tests at or below the concentrations given: 450 ppm Cu_2O, 500 ppm $CuCl_2$, and 2 ppm Puratized. Spore formation of *Glomerella* R strains in the plug test was

decreased by these compounds in the order given: Bioquin-1, Bordeaux, ferbam, Cu_2O, $CuCl_2$, and Puratized. Bioquin and $CuSO_4$ R strains lost the ability to form spores and apparently mutated. Isothan DL-1 R strain produced as many spores as the C-1 strain.

Glomerella R strains developed resistance to a significant degree in the plug tests at or below 475 ppm Cu_2O, 600 ppm $CuCl_2$, and 5 ppm Puratized. Resistance was carried over to the spores exposed to the following chemicals: for Cu_2O the LD 90 of the R and C strains was 10 and 13 ppm respectively, for ferbam the LD 50 of the R and C strains was 0.08 and 0.02 ppm respectively. Resistance developed in the $CuSO_4$ exposed R strain by the formation of more aerial growth than the C strain.

Glomerella R strains mutated when exposed to Cu_2O, $CuSO_4$, $CuCl_2$, Bioquin, and ferbam. This was shown when the R strains did not revert to the C-1 strain after transfer to inhibitor-free medium. They differed from the control in color, lack of spores in $CuSO_4$ and Bioquin exposed R strains, size of spores in the other R strains, size of mycelium, and texture of mycelial mat. When inoculated into apple the R strain from the above 5 chemicals failed to produce spores and grew less than the C-1 strain. The average growth rates of the R and C-1 strains on inhibitor-free medium are shown in the table. The mutant on Cu_2O decreased in aerial mycelial growth.

Sectors of *Glomerella* appeared occasionally in the control cultures. These had white to nearly black mycelial growths. The R strain exposed to Puratized produced a definite sector at 2.5 ppm which outgrew the rest of the colony. Transfers from this sector showed a definite resistance had developed. Ferbam R strain developed a white sector in about ¼ of the transfers but was not resistant. After several transfers to the chemicals the mutated R strains generally did not change any further.

Penicillium exposed to chemicals followed somewhat the same pattern of growth and spore inhibition as the two foregoing fungi. *Penicillium* apparently developed resistance in the mass spore tests to the following chemicals at the concentrations indicated: 280 ppm Cu_2O, 600 ppm $CuSO_4$, 500 ppm $CuCl_2$, 17.5 ppm Bioquin. Decreasing sporulation of the R and C strains occurred as follows: C-1, Isothan DL-1, Bioquin, Bioquin-1, ferbam, Puratized, Bordeaux, $CuSo_4$, $CuCl_2$, and Cu_2O. No apparent difference could be noted between R and C-1 strains after growth on apple following 10 transfers to inhibitor-free medium. All produced good growth and heavy sporulation. The R strains from Bioquin-1, Bioquin, and ferbam did not revert to the parent type on inhibitor-free medium.

Penicillium developed resistance in the plug tests to a significant

degree on the following chemicals at or below the concentrations given: 260 ppm Cu_2O, 750 ppm $CuSO_4$, 550 ppm $CuCl_2$, and 30 ppm Bioquin. Resistance was carried over to the spores exposed to the following chemicals: for $CuSO_4$ the LD 90 of the R and C strains was 12 and 6.7 ppm respectively; for Bioquin-1 the LD 90 of the R and C strains was 2.9 and 1.9 respectively; and for $CuCl_2$, some resistance was developed.

Although changes occurred in the R strains exposed to some of the chemicals, these changes were not enough to differentiate them from the *Penicillium expansum* type. Some of the changes were permanent and were substantiated by repeating the tests with some of the chemicals. Bioquin and some of its derivatives resulted in changes similar to those in the first tests. The average growth rates of the R and C-1 strains on inhibitor-free medium are given in the table.

Many of the changes induced by the chemicals were in the loss or increase of spore formation; color changes as in *Glomerella* R strains on Bioquin and ferbam, both causing the production of soluble orange pigment; size of spores reduced as *Glomerella* on Cu_2O, $CuCl_2$, and ferbam. Ropy and uniformly thin mycelial strands occurred in the mutated R strains of *Glomerella* and colonies entirely of coremia occurred in Bioquin-1 and ferbam R strains of *Penicillium*.

In Method II *Sclerotinia* developed resistance to Bordeaux at or below 168 ppm, Bioquin-1 6 ppm, and ferbam 60 ppm. Resistance did not develop in *Glomerella* or *Penicillium*. However there was a trend toward development of resistance in the dry and ash weights. The chemicals apparently had little effect on the pH of the Czapek's medium in the flasks. From the results obtained it was apparent that *Sclerotinia* developed resistance more easily than the other two fungi. *Glomerella* produced the greatest number of variations while *Penicillium* was intermediate between the other two fungi. All the chemicals showed a reduction in their toxic effect against the three fungi when added to the whole-wheat agar. This was due to the adsorption of the chemicals by the medium. An example of the quantity adsorbed was shown by the action of ferbam on spores of *Penicillium* in medium. Spore germination barely occurred at 125 ppm, while in 1 per cent apple juice 1.6 ppm allowed germination. The effects varied with the fungus and chemical. In Method II Puratized R strain of *Glomerella* hydrolyzed starch after growth in Czapek's medium.

Of the two methods used the plate-plate-plug transfers of Method I was the best. Other workers have found that spores were more susceptible to the action of the toxicants than the mycelium. The use of a number of variables as in Method II does not hasten the development of resistance.

IV. Summary

In conclusion and in answer to the questions asked at the beginning it may be said that: 1. Resistance can be developed to certain fungicides. There is evidence to show that this resistance can be repeated. 2. There was evidence to show that mass inoculum in the form of plugs of mycelium and spores could be used in developing resistance. 3. The effects of the toxic agent were never completely overcome. 4. Permanent morphological and probably biochemical changes occurred as mutations. 5. It was shown that the viability of certain R strains was changed. 6. The fact that resistance developed and mutations occurred when the fungi were exposed to certain commercial fungicides gives some indication that the same changes may occur in the field although no evidence has been presented on this point. 7. There was evidence that the concentration of the chemicals had little to do with development of resistance or the mutations. The time factor was proven when *Penicillium* was exposed to Bioquin for 10 transfers before it changed. Then when this was repeated the same change occurred after the second transfer. Other instances of this occurred during the course of the experiments.

VITA

Omar Drennan Morgan, Jr., was born March 5, 1913, in Shelbyville, Mo. Public school education in East Peoria, Ill., was followed by a B.Ed. degree with major in biology from the Illinois State Normal University in 1940. The next year he held a teaching assistantship in agricultural and general botany at the University of Arkansas, and the following year taught science in the Community High School at Cullom, Ill.

From 1942-1944 as an enlisted Naval Reserve he was trained as a medical laboratory technician at Great Lakes, Ill., and as epidemiologist at the Naval Hospital in Bethesda, Md. He was commissioned a Lt. (jg) DL in 1944 and served in the Central Pacific area in 1945.

In 1946 he resumed his education at the University of Illinois Graduate College, majoring in plant pathology. He held a Monsanto fellowship in fungicide research his last two years as a graduate student. Before completing his work for the degree of Doctor of Philosophy, he received a position as assistant professor in plant pathology at the University of Maryland.

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His publications are:

Morgan, Omar D., Jr., and Dwight Powell. Specificity of certain Bioquin (8-quinolinol) derivatives and some of their formulations for apple scab control. Abst., Phytopath. 38: 1948.

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